Molecular and functional consequences of Smad4 C-terminal missense mutations in colorectal tumour cells

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Smad4 is an essential signal transducer of the transforming growth factor β (TGF-β) signalling pathway and has been identified as a tumour suppressor, being mutated in approx. 50 % of pancreatic cancers and approx. 15 % of colorectal cancers. Two missense mutations in the C-terminal domain of Smad4, D351H ($Asp^{351} \rightarrow$ His) and D537Y ($Asp^{537} \rightarrow$ Tyr), have been described recently in the human colorectal cancer cell lines CACO-2 and SW948 respectively [Woodford-Richens, Rowan, Gorman, Halford, Bicknell, Wasan, Roylance, Bodmer and Tomlinson (2001) Proc. Natl. Acad. Sci. U.S.A. **98**, 9719–9723]. Previous work *in vitro* suggested that only Asp-351 was required for interaction with Smad2 [Wu, Fairman, Penry and Shi (2001) J. Biol. Chem. **276**, 20688–20694]. In the present study, we investigate the functional consequences of these point mutations *in vivo*. We demonstrate that neither of these colorectal cancer cells undergo growth arrest in response to TGF-β, which can be explained, at least in part, by their inability to up-regulate cyclin-dependent kinase inhibitors $p21^{CIP1}$ or $p15^{INK4b}$ after TGF-β stimulation. Although the point-mutated Smad4s are expressed at normal levels in these colorectal cancer cells, they cannot interact with either TGF-β-induced phosphorylated Smad2 or Smad3. As a result, these Smad4 mutants do not accumulate in the nucleus after TGF-β stimulation, are not recruited to DNA by relevant Smad-binding transcription factors and cannot generate transcriptionally active DNA-bound complexes. Therefore both these colorectal tumour cells completely lack functional Smad4 activity owing to the missense mutations. Given the location of these mutations in the three-dimensional structure of the Smad4 C-terminal domain, the results also give us significant insights into Smad complex formation.

Key words: colorectal cancer, Smad complex, Smad4, TGF-β (transforming growth factor β) signalling, tumour suppressor, tumorigenesis.

INTRODUCTION

Transforming growth factor β (TGF-β) is a pleiotropic cytokine that regulates a large number of cellular functions, including cell migration, adhesion, proliferation, differentiation and programmed cell death. It elicits these cellular responses by binding to a heteromeric receptor complex at the cell surface [1]. These receptors, which are serine/threonine kinases, propagate the signal by phosphorylating and activating two members of the Smad family, namely the receptor-regulated Smads (R-Smads) Smad2 and Smad3 [1]. The phosphorylated Smad2 and Smad3 associate with the co-Smad Smad4, forming complexes that accumulate in the nucleus and regulate the transcription of TGF-β-responsive genes. Smads are modular proteins consisting of a very well-conserved N-terminal MH1 (Mad homology 1) domain, which for Smad3 and Smad4, binds DNA, a variable flexible proline-rich linker region and a very well-conserved C-terminal MH2 domain required for Smad–Smad interaction and transcriptional activation [1].

Recent work has indicated that the Smads shuttle between the cytoplasm and nucleus in both the absence and presence of TGF-β [2,3]. In unstimulated cells, Smad4 shuttles between the cytoplasm and the nucleus as a result of a constitutively active nuclear localization signal in its MH1 domain and a constitutively active nuclear export signal in its linker region, whose activity depends on the nuclear export protein CRM1 [4–6]. The accumulation of Smad4 in the nucleus after TGF-β stimulation is thought to be due to complex formation with activated R-Smads [6,7]. Smad2 (and probably also Smad3) can also shuttle between the nucleus and the cytoplasm in unstimulated cells [3]; the predominant cytoplasmic localization of these Smads in uninduced cells presumably reflects the fact that nuclear export is dominant over nuclear import. The Smads are also constantly shuttling between the cytoplasm and nucleus during active signalling and thereby continuously monitor the levels of receptor activity [2].

In the nucleus, the Smads act in conjunction with other transcription factors to regulate the transcription of target genes [1]. Smad2–Smad4 complexes are recruited to DNA by transcription factors, such as forkhead/winged-helix proteins of the FoxH1 family, or the paired-like homeodomain proteins of the Mix family, such as Mixer [8,9]. Smad3–Smad4 complexes can bind DNA directly and recognize the Smad-binding element 5′-GTCT-3′. However, to form stable complexes on DNA, they appear to also require association with other transcription factors [10]. Active Smad complexes may be either heterodimers or heterotrimers [10]. The Smad2–Smad4 complex that associates *in vivo* with FoxH1 family members at the ARE (activin-responsive element) from the *Xenopus Mix.2* promoter is trimeric, consisting of two Smad2 subunits and one Smad4 subunit. In contrast, the Smad3–Smad4 complex that binds the SBR (Smad binding region) of the human c-*Jun* promoter is a dimer and contains at least two additional components [10].

Aberrant TGF-β signalling has been strongly implicated in cancer and it plays a complex role [11]. At early stages, TGF-β acts as a tumour suppressor, thought to be mainly owing to its ability to induce growth arrest and apoptosis of epithelial cells. As tumours progress, they become resistant to the tumour-suppressive

Abbreviations used: ARE, activin-responsive element; ARF1, activin-responsive factor 1; HA, haemagglutinin; IP, immunoprecipitation; LMB, leptomycin B; MH1, Mad homology 1; R-Smads, receptor-regulated Smads; SBR, Smad binding region; TGF-β, transforming growth factor β.

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effects of TGF-β, but maintain responsiveness and frequently secrete TGF-β. At later stages, TGF-β acts as a tumour promoter by stimulating invasiveness and metastasis [11]. Consistent with its tumour suppressor role, several components of the TGF-β signalling pathway have been found to be mutated or deleted in human tumours: in particular, the type II receptor, which is mutated in colon and gastric tumours as a result of defects in DNA replication error repair, and Smad4, which is mutated or deleted in about half the pancreatic tumours and approx. 15 % of colorectal tumours [11].

The role that loss of Smad4 function plays in the progression of human tumours is not entirely clear. Even though Smad4 is thought to be a central component of the pathway, Smad4-null tumour cell lines appear to retain at least some TGF-β responses, although the mechanism underlying Smad4-independent signalling is not fully understood [11–14]. In some cases, loss of Smad4 is associated with specific loss of TGF-β-induced growth arrest [11,15]. In other cases, loss of Smad4 in tumours has been shown to be important for increasing angiogenesis and for loss of epithelial integrity [16,17].

Loss of Smad4 in human tumours can occur by loss of the entire chromosome region, small deletions, frameshifts, nonsense or missense mutations [18]. Analysis of these naturally occurring Smad4 mutants is an important step towards understanding the mechanism of action of Smad4 and also its biological role. In the present study, we have focused on two different naturally occurring point mutations in the MH2 domain of Smad4, namely D351H and D537Y, which were identified in the colorectal cancer cell lines CACO-2 and SW948 respectively [19]. We have studied the functional consequences of these mutations. Both these tumour cells have also lost the wild-type allele of Smad4 and thus only express the mutated allele [19]. We show, using a variety of *in vivo* assays in the CACO-2 and SW948 cells themselves and in heterologous systems, that both of these point-mutated Smad4s are functionally inactive in the tumour cells, as a result of their inability to interact with either activated Smad2 or Smad3. This contrasts with conclusions drawn previously from *in vitro* studies of these mutants [20,21], emphasizing the importance of studying Smad function *in vivo*. Given the location of these mutations in the three-dimensional structure of the Smad4 C-terminal domain, the results also provide important insights into Smad complex formation.

EXPERIMENTAL

Plasmids and reagents

The following plasmids have been described previously: ARE_3-luciferase, EF-plink, hSmad4 and XSmad2 in an HA (haemagglutinin)- or FLAG-tagged EF expression vector [5,9], XFoxH1a (previously known as XFast-1) in an EF-FLAG expression vector [9], $CAGA_{12}$-luciferase [22] and EF-LacZ [23]. The point-mutated versions of hSmad4 in the EF-HA expression vector, EF-HA-Smad4 D351H and EF-HA-Smad4 D537Y were generated by PCR and checked by sequencing.

TGF-β1 (Peprotech, Rocky Hill, NJ, U.S.A.) was dissolved in 4 mM HCl and 1 mg/ml BSA, and used at a final concentration of 2 ng/ml. Leptomycin B (LMB) was resuspended in ethanol at a concentration of 10 μg/ml and used at a final concentration of 20 ng/ml.

Cell lines, transfection and transcriptional assays

All the cell lines used were described previously: SW948 and CACO-2 [19], HeLa-TK$^-$ [24] and MDA-MB468 [25]. They were grown in Dulbecco's modified Eagle's medium/10 % (v/v) foetal calf serum, except for the SW948 cell line which was grown in Iscoves modified Dulbecco's medium/10 % foetal calf serum. HeLa-TK$^-$ cells were transfected with LIPOFECTAMINE™ (Invitrogen) and MDA-MB468 cells with Superfect Reagent (Qiagen), both according to the manufacturer's instructions, using the plasmids indicated in the Figure legends. All cells were analysed 24 h after transfections. Cells were induced with 2 ng/ml TGF-β1 for 8 h and luciferase assays were performed as described previously [5]. β-Galactosidase assays were performed using Galactostar (Applied Biosystems, Foster City, CA, U.S.A.) and analysed in a luminometer as for luciferase.

Cell-cycle analysis

Cells growing at low density (40 %) in normal growth medium were left untreated or treated with 2 ng/ml TGF-β1 for 24 h. Cells were then trypsinized and fixed with 70 % ethanol. They were then washed twice with PBS, treated with RNase A (100 ng/ml) and stained with 50 μg/ml propidium iodide for flow-cytometric analysis.

RNA isolation and RNase protections

Cells grown to a 70–80 % density were stimulated with TGF-β1 for different lengths of time. Cell lysis, RNA extraction, probe preparation and RNase protection assays were performed as described previously [5]. All the probes used were prepared as described in [26].

Immunoprecipitation, Western-blot analysis and bandshift assays

Whole cell and nuclear extracts were prepared as described in [9,27]. Western-blot analysis was performed using standard techniques. The following antibodies were used: anti-Smad2/3 (BD Biosciences, San Jose, CA, U.S.A.), anti-Smad4 (B8; Santa Cruz Biotechnology) and anti-phosphorylated Smad2 [28]. Immunoprecipitation (IP) and Western-blot analysis were performed as described in [5], and the rabbit polyclonal anti-Smad2 antibody used for IP was described previously [29]. The immunoprecipitates were probed with the mouse monoclonal anti-Smad2/3 and anti-Smad4 antibodies described above.

Bandshift assays using nuclear extracts and the probe corresponding to the SBR from the c-*Jun* 5′-UTR were as described in [10]. Bandshift assays using whole cell extracts and the probe corresponding to the ARE were as described in [9]. Supershift analyses were performed with 1 μl of an appropriate antibody. The anti-Smad2/3 and anti-Smad4 antibodies were as described above; anti-HA was obtained from Roche Molecular Biochemicals.

Confocal fluorescence microscopy and light microscopy

Detection of Smad2/3 or Smad4 by immunostaining was as described in [5]. Samples were analysed using a Zeiss confocal LSM 510 microscope.

RESULTS

The colorectal cancer cell lines CACO-2 and SW948 do not undergo growth arrest on TGF-β stimulation

We first investigated to what extent the presence of a Smad4 containing a point mutation in its MH2 domain affected the TGF-β responsiveness of CACO-2 and SW948 cells. As a positive control, we used the human keratinocyte cell line HaCaT, which contains functional wild-type Smad4.

In general, cells of epithelial origin undergo growth arrest in the G_1-phase of the cell cycle on TGF-β treatment [18]. We compared

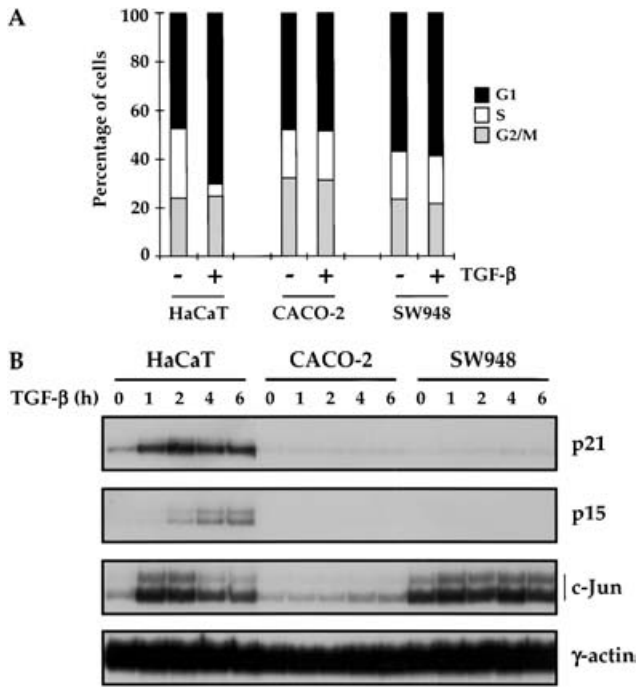

Figure 1 SW948 and CACO-2 cells are resistant to TGF-β-induced growth arrest

(**A**) TGF-β induction of cell-cycle arrest in various cell lines. Actively growing low-confluency cells (40–50 %) were either untreated or treated with 2 ng/ml TGF-β1 for 24 h. Samples were analysed by FACS to determine the number of cells in G_1-phase (black bar), S-phase (white bar) or G_2/M-phase (grey bar). A representative experiment is shown. (**B**) The induction of specific TGF-β-responsive target genes is perturbed in CACO-2 and SW948 cell lines. Total RNA purified from different cell lines, untreated or treated with 2 ng/ml TGF-β1 for the indicated time periods, was assayed for the expression levels of $p21^{WAF1/Cip1}$ (*p21*), $p15^{INK4B}$ (*p15*), c-*Jun* and γ-actin (loading control) by RNase protection. Protected fragments are indicated.

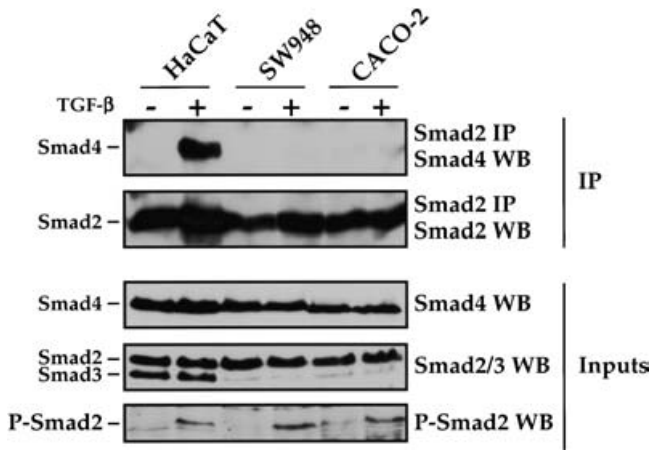

Figure 2 Endogenous Smad4 D351H and D537Y do not interact with activated Smad2 in CACO-2 and SW948 cells respectively

Cells were untreated or treated with 2 ng/ml TGF-β for 1 h, after which whole cell extracts were immunoprecipitated (IP) with a rabbit polyclonal anti-Smad2 antibody, followed by Western-blotting with a mouse monoclonal antibody against Smad4 or Smad2/3 (IP). As a control for the input before IP, 30 μg of the whole cell extract was analysed by Western-blot analysis with monoclonal antibodies against Smad4 and Smad2/Smad3 or with a polyclonal antibody against phosphorylated Smad2 (P-Smad2) (inputs).

the cell-cycle profiles of the colorectal cancer cell lines CACO-2 and SW948 with those of the HaCaT cells, in the absence or presence of TGF-β stimulation (Figure 1A). As expected, HaCaT cells accumulated in G_1-phase in response to TGF-β. However, the percentage of cells in G_1-, S- or G_2/M-phase did not change in SW948 or CACO-2 cells on treatment with TGF-β, indicating that neither of these cell lines exhibited a TGF-β-induced growth arrest.

To investigate this further, we performed RNase protection assays to test the TGF-β inducibility of genes known to be involved in TGF-β-dependent growth arrest, focusing on the CDK inhibitors $p21^{WAF1/Cip1}$ (hereafter *p21*) [30,31] and $p15^{INK4B}$ (hereafter *p15*) [32]. We also investigated the inducibility of the AP-1 transcription factor c-*Jun* [33], which is implicated in the autocrine production of TGF-β [34].

Both *p21* and *p15* were induced in HaCaT cells and their inductions were sustained for at least 6 h after TGF-β stimulation (Figure 1B). *p21* induction was strong and it was detected within 1 h, whereas *p15* induction was weaker and detected only after 2 h as demonstrated previously [26]. Neither of the colorectal cancer cell lines showed an up-regulation of either *p21* or *p15*. A strong TGF-β-dependent c-*Jun* induction was visible after 1 h in HaCaT cells, reaching a peak at 2 h and decreasing after 4 h. Neither of the colorectal cancer cell lines showed a TGF-β-dependent regulation of c-*Jun* with time, although a high basal expression of c-*Jun* was observed in the SW948 cells.

Thus neither of the colorectal cancer cell lines that contain point-mutated Smad4 undergo growth arrest in response to TGF-β. Consistent with this, these cells have lost the ability to regulate the genes required for growth arrest on TGF-β stimulation. They also fail to regulate the expression of c-*Jun* in response to TGF-β.

The point-mutated Smad4s do not interact with activated Smad2 in the colorectal cancer cell lines CACO-2 and SW948

Previous work has indicated that both Asp-351 and Asp-537 in Smad4 were essential *in vitro* for the stability of the Smad4 homotrimeric complex [35], although only Asp-351 was essential for interaction with Smad2 *in vitro* [20,21]. However, the presence of these mutations in Smad4 in tumour cells that have lost TGF-β responses suggests that both of these residues are critical for Smad4 function. We therefore investigated the Smad signalling pathway in these cell lines in detail, first testing whether the point-mutated Smad4s could interact with activated Smad2 *in vivo*.

We performed an IP with an anti-Smad2 antibody using total cell extracts prepared from HaCaT, SW948 and CACO-2 cells treated with or without TGF-β for 1 h and then Western-blotted the immunoprecipitated proteins for either Smad4 or Smad2 (Figure 2). Only wild-type Smad4 from TGF-β-induced HaCaT cells was capable of forming a strong complex with activated Smad2. The point-mutated Smad4 variants from CACO-2 and SW948 cells did not interact with Smad2 on TGF-β stimulation. The control showed that, in all cases, approximately equal amounts of Smad2 were immunoprecipitated. Importantly, blotting of total cell extracts (inputs) demonstrated that all the cell lines had equivalent levels of Smad2 and Smad4 (although levels of Smad3 were quite low in the CACO-2 and SW948 cell lines; Figure 2, lower panels). Furthermore, TGF-β-induced phosphorylation of Smad2 was observed in all the cell lines, demonstrating that TGF-β signalling at the level of the TGF-β receptors was not altered in these colorectal cancer cell lines.

Thus TGF-β-induced phosphorylation of Smad2 is normal in these tumour cell lines and the levels of Smad4 are normal. However, no interaction occurred between activated Smad2 and Smad4 as a result of the point mutations in Smad4. Therefore both amino

acids Asp-351 and Asp-537 are essential *in vivo* for the interaction of Smad4 with activated Smad2.

Smad4 D351H and D537Y variants do not form transcriptionally active DNA-binding complexes with activated Smad2 and XFoxH1a

Our results indicate that endogenous point-mutated Smad4 could not interact with TGF-β-activated Smad2 *in vivo* in CACO-2 or SW948 cells. It was possible, however, that these Smad4 mutants might still be physically recruited into the transcriptionally active complexes, such as ARF1 (activin-responsive factor 1) which binds the ARE [8] and contains XFoxH1a (previously known as XFast-1), Smad2 and Smad4. This is because, in addition to Smad2–Smad4 interactions, formation of this complex also requires the direct interaction of the Smad4 MH1 domain with DNA [36,37], which is not affected by the point mutations. We therefore investigated whether these mutated Smad4s could be incorporated into an ARF1 complex by bandshift analysis. Since CACO-2 and SW948 cells do not transfect efficiently, we performed this experiment in HeLa TK$^-$ cells, transfected with XFoxH1a and/or FLAG-Smad2 and/or HA-Smad4 variants and treated with TGF-β for 1 h. A TGF-β-induced ARF1 complex containing endogenous Smad2 and Smad4 was detected when XFoxH1a was overexpressed (lanes 4 and 5 in both Figures 3A and 3B). When HA-tagged Smad4 was overexpressed, this protein clearly replaced some of the endogenous Smad4, as evidenced by the partial supershift obtained with an anti-HA antibody (lanes 8 and 9 in both Figures 3A and 3B). However, when HA-tagged Smad4 D537Y or D351H variant was expressed, no supershifted complex appeared when the anti-HA antibody was added to the bandshift reaction (lanes 11 and 12 in both Figures 3A and 3B). This indicates that whereas wild-type HA-tagged Smad4 can bind activated Smad2 and XFoxH1a at the ARE, point-mutated Smad4s cannot. Introducing excess FLAG-tagged Smad2 to maintain a balance of Smad2 and Smad4 in the cell yielded the same overall results (lanes 13–18 in both Figures 3A and 3B). Thus neither of the Smad4 point mutants can be incorporated into a DNA-bound ARF1 complex.

We confirmed this result using a much more sensitive reporter assay. MDA-MB468 cells were used for these assays, as they lack Smad4 [25], and we could thus test the ability of the Smad4 point mutants to rescue this Smad4 deletion. We used a reporter driven by three copies of the ARE from the *Mix.2* promoter that binds a Smad2–Smad4 complex in association with XFoxH1a [9]. When XFoxH1a alone was transfected, a low TGF-β inducibility was observed, which is probably due to the recruitment of endogenous activated Smad2 to the XFoxH1a (Figure 3C) [5]. Introducing wild-type Smad4 in MDA-MB468 cells strongly enhanced the basal level of XFoxH1a-dependent transcription and gave an extra 2-fold induction after TGF-β stimulation. However, when the Smad4 point-mutated variants were transfected, this did not result in any significant additional transactivation (Figure 3C). Similar levels of expression for the tagged Smad4 variants were confirmed by Western-blot analysis (results not shown). Thus neither Smad4 D351H nor Smad4 D537Y can participate in the formation of functional transcription complexes with activated Smad2.

The Smad4 variants found in SW948 and CACO-2 do not form functional transcriptional complexes with activated Smad3

We next investigated the interaction between the point-mutated Smad4s and activated Smad3 by testing whether an endogenous Smad3–Smad4 DNA-bound complex was formed on the SBR of the c-*Jun* 5′-UTR [10,27] when cells were stimulated with TGF-β. The levels of Smad3 in CACO-2 and SW948 cells

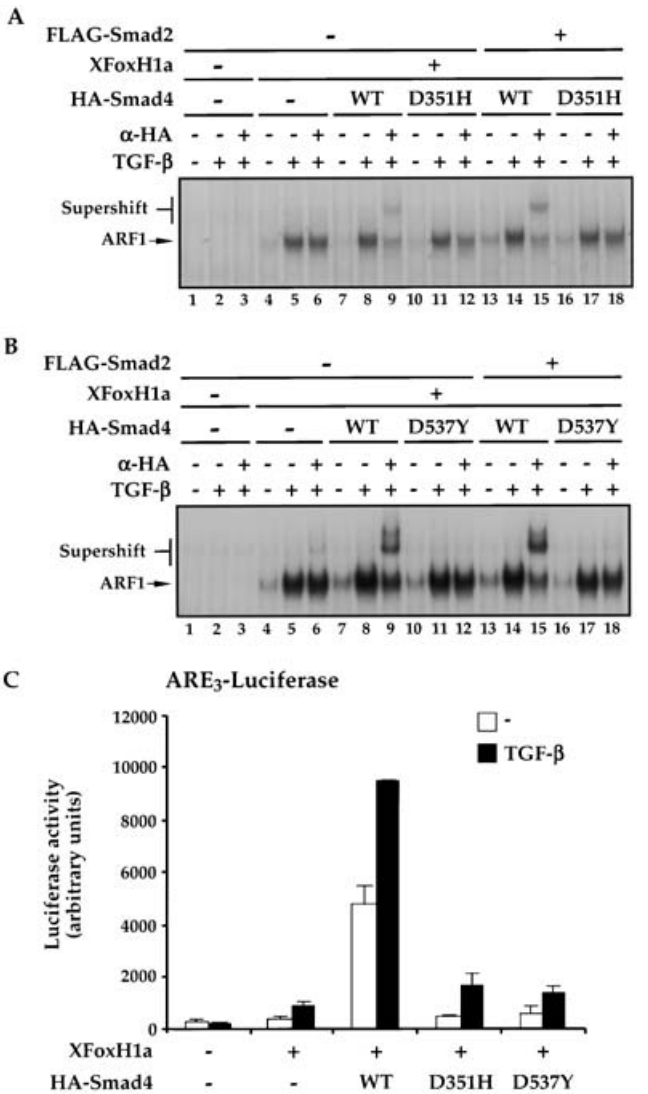

Figure 3 The point-mutated Smad4s do not form transcriptionally active DNA-binding complexes with activated Smad2 and XFoxH1a

(**A**, **B**) HeLa TK$^-$ cells were transfected with different combinations of expression plasmids EF-FLAG-XFoxH1a, EF-FLAG-Smad2 and EF-HASmad4, EF-HASmad4D351H (**A**) or EF-HASmad4D537Y (**B**) as indicated. Whole cell extracts were prepared from cells that were either untreated or treated with 2 ng/ml TGF-β1 for 1 h and analysed by a bandshift assay using ARE as a probe. The ARF1 complex is indicated (arrow) as are the anti-HA supershifted complexes. (**C**) MDA-MB468 cells were transfected with ARE$_3$-luciferase together with EF-LacZ, EF-FLAG-XFoxH1a and EF-HASmad4 or the point-mutated variants EF-HASmad4D351H or EF-HASmad4D537Y as indicated. Cells were either untreated or treated with TGF-β for 8 h. Luciferase was quantified relative to β-Gal from the EF-LacZ internal control. Results are the means $\pm$ S.D. for a representative experiment performed in quadruplicate.

are quite low, but we have shown previously that it is possible to detect this complex in cells that contain very low levels of Smad3 [26]. No Smad3–Smad4 complexes were formed on the SBR probe using extracts from TGF-β-stimulated SW948 or CACO-2 cells (Figure 4A); this is in contrast with HaCaT cells where TGF-β induction resulted in the formation of a DNA-bound Smad3–Smad4 complex. The presence of both Smad3 and Smad4 in these complexes was confirmed by supershift analysis using anti-Smad2/3 (which recognizes both Smad2 and Smad3) and anti-Smad4 antibodies (Figure 4A). These results suggest that the Smad4 point mutations prevent the formation of a Smad3–Smad4 DNA-bound complex.

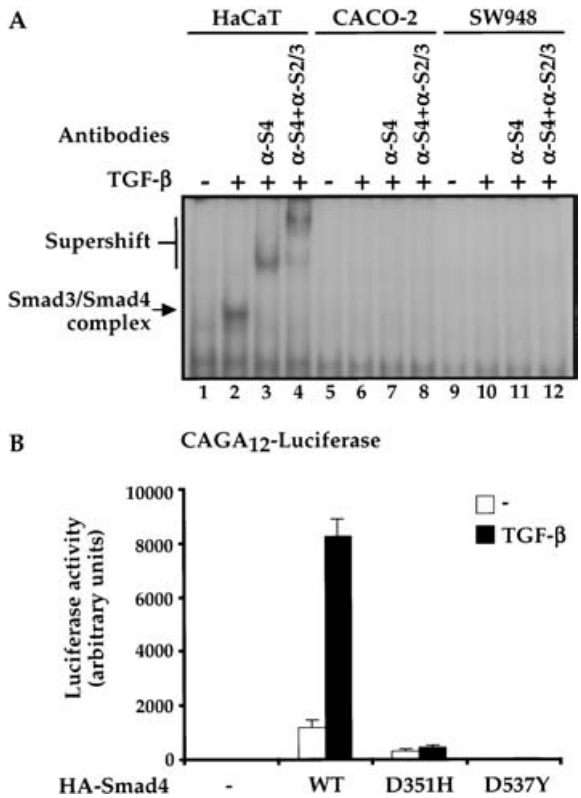

Figure 4 The point-mutated Smad4s do not form a transcriptionally active DNA-bound Smad3–Smad4 complex in response to TGF-β

(**A**) HaCaT, SW948 and CACO-2 cells were either untreated or treated for 1 h with 2 ng/ml TGF-β1. Nuclear extracts were prepared and assayed by bandshift analysis using the c-*Jun* SBR as a probe. The arrow indicates the position of the endogenous Smad3–Smad4 complex, as found in extracts from control HaCaT cells only. Supershifts (indicated) were performed with an anti-Smad4 (α-S4), and anti-Smad4 and anti-Smad2/Smad3 (α-S2/S3 + α-S4) antibodies. (**B**) MDA-MB468 cells were transfected with either $CAGA_{12}$-Luciferase together with EF-LacZ and EF-HASmad4 or the point-mutated variants, EF-HASmad4D351H or EF-HASmad4D537Y as indicated. Cells were either untreated or treated with TGF-β for 8 h. Luciferase was quantified relative to β-Gal from the EF-LacZ internal control. Results are the means $\pm$ S.D. for a representative experiment performed in quadruplicate.

We confirmed this result using a more sensitive reporter assay. Again MDA-MB468 cells were used for this experiment, as CACO-2 and SW948 cells do not transfect efficiently. The reporter used was $CAGA_{12}$-luciferase reporter, which is driven by 12 copies of the 'CAGAC' sequence derived from the *PAI-1* promoter and is activated by Smad3–Smad4 complexes [22]. This reporter is inactive in MDA-MB468 cells, as they lack Smad4 (Figure 4B). A strong TGF-β-inducible transactivation of the $CAGA_{12}$-luciferase reporter was detected when wild-type Smad4 was expressed, but not when either of the mutant variants of Smad4 were expressed (Figure 4B). This confirms that the mutant Smad4s cannot interact with activated Smad3. Similar levels of expression for the tagged variants of Smad4 were confirmed by Western-blot analysis (results not shown).

Thus, taken together with the results of the previous section, our results demonstrate that neither Smad4, D537Y nor Smad4 D351H can interact with activated Smad2 or Smad3.

Endogenous Smad4 D351H and D537Y do not accumulate in the nucleus after TGF-β stimulation

We have shown in a variety of assays that the point-mutated Smad4s do not interact with activated Smad2 or Smad3. We finally tested what effect this had on the subcellular distribution of Smad4 in the presence or absence of TGF-β and in the presence of the CRM1 inhibitor LMB by detecting the endogenous Smads in SW948, CACO-2 and HaCaT cells by immunostaining (Figure 5). Before TGF-β treatment, all three Smad4 variants (wild-type, D351H and D537Y) were similarly distributed between the cytoplasm and nucleus (Figure 5, left panels). On stimulation with TGF-β for 1 h, wild-type Smad4 accumulated in the nucleus (HaCaT cells). In contrast, the distribution of Smad4 D351H or D537Y did not change (CACO-2 and SW948 cells), suggesting that complex formation with activated R-Smads is required for Smad4 to accumulate in the nucleus in response to TGF-β. To investigate this further, we tested whether nucleocytoplasmic shuttling of Smad4 itself was affected by the mutations. When unstimulated HaCaT cells were treated with LMB, wild-type Smad4 accumulated in the nucleus (Figure 5, left panels), since it is constitutively imported into the nucleus, but is prevented from being exported owing to the inhibition of CRM1 by LMB [5]. Similarly, the two mutated Smad4s also accumulated in the nucleus on LMB treatment. This indicates that the Smad4 mutants are recognized by the transport machinery and are not defective in nucleocytoplasmic shuttling.

As a control, we investigated the behaviour of Smad2 and Smad3 in the three different cell lines using an antibody that recognizes both Smad2 and Smad3 (Figure 5, right panels). Induction with TGF-β led to the nuclear accumulation of Smad2–Smad3 in all three cell lines, whereas treatment with LMB had no effect on the localization of these R-Smads. Thus the presence of the mutations in Smad4 did not interfere with the activation of Smad2–Smad3 in response to TGF-β.

Taken together, these results show that on TGF-β induction, activated Smad2 and Smad3 are capable of translocating to the nucleus and reside there independently of Smad4. In contrast, and importantly, we show that Smad4 must interact with activated Smad2 or Smad3 to be retained in the nucleus on TGF-β stimulation, although not to shuttle between the cytoplasm and nucleus in unstimulated cells.

DISCUSSION

Smad4 is frequently mutated in colon and pancreatic carcinomas [11]. In these tumours, Smad4 mutations appear more frequently in the MH2 domain, and may be missense or nonsense mutations [19,25,35,38]. The reported nonsense mutation at amino acid 515 results in an unstable protein [39]; the missense mutations have been reported to affect the ability of Smad4 to form complexes with R-Smads *in vitro* [35]. Other mutations have been found in the N-terminal MH1 domain; they cause protein instability, owing to a higher susceptibility to ubiquitin-mediated degradation, and can also affect DNA binding or nuclear translocation [4,40,41].

In the present study, we have investigated the molecular basis and functional consequences for TGF-β signalling of the missense point mutations D351H and D537Y in the Smad4 MH2 domain that occur naturally in the human colorectal tumour cell lines CACO-2 and SW948 respectively [19]. The structure of the monomeric C-terminal MH2 domain of Smad4 consists of a β-sandwich; one of its ends is capped by a three-helix bundle (containing α-helices 3, 4 and 5), whereas the other is capped by a group of three large loops and an α-helix, referred to as the loop–helix region (Figure 6) [35]. Residue Asp-351 in Smad4 occurs in the loop–helix region and Asp-537 occurs on α-helix 5 in the helix bundle region (Figure 6) [35]. Mutation of either residue disrupts the ability of the Smad4 MH2 domain to form homotrimers [35]. In contrast, only mutation of Asp-351 was shown *in vitro*

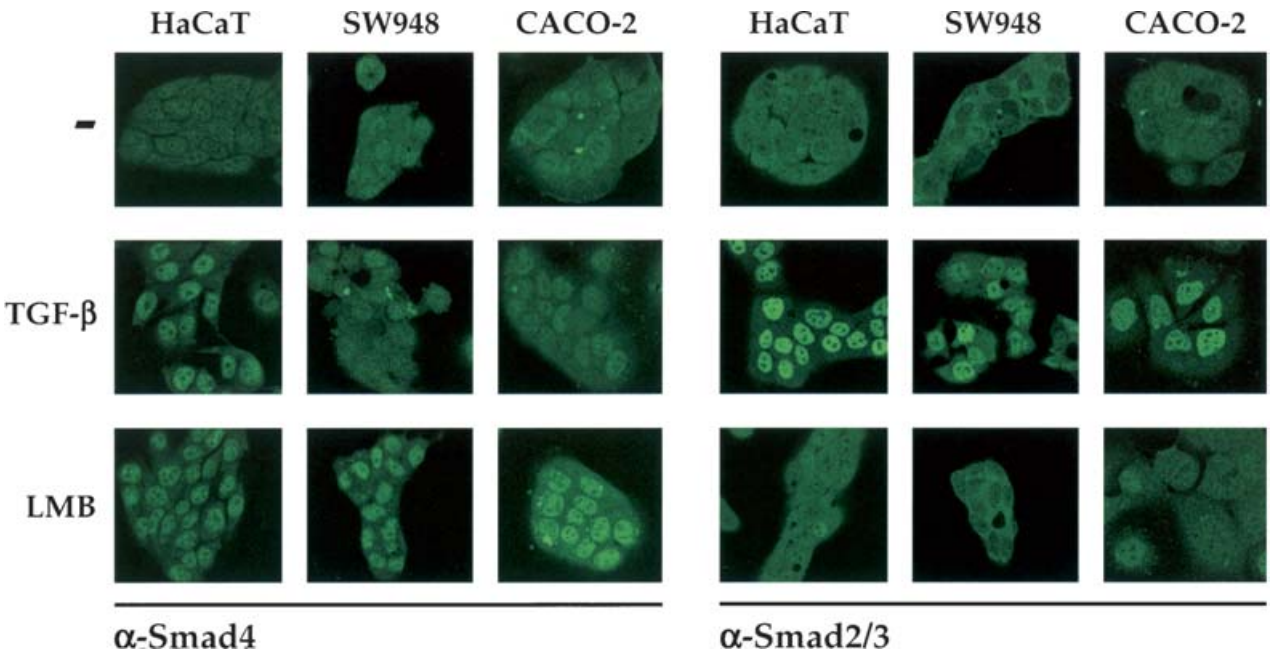

Figure 5 TGF-β-induced Smad4 nuclear translocation is impaired in colorectal cancer cells with point-mutated Smad4 variants

HaCaT, CACO-2 or SW948 cells were either untreated or treated with 2 ng/ml TGF-β1 or 20 ng/ml LMB for 1 h. Samples were fixed, processed for immunofluorescence using an anti-Smad2/Smad3 or an anti-Smad4 antibody, as indicated, and examined by confocal laser scanning microscopy.

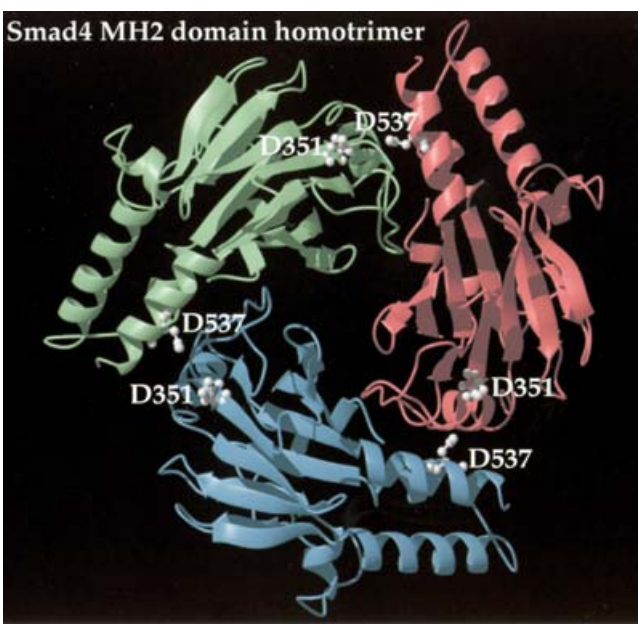

Figure 6 The structure of the homotrimer of Smad4 MH2 domains

The structure of the Smad4 MH2 domain homotrimer is shown indicating residues Asp-351 and Asp-537, which form part of a hydrogen-bonding network at the interface between Smad monomers. The Figure was reconstructed from the crystal co-ordinates of the Smad4 MH2 domain structure [35] using the programme MOLMOL [42].

to prevent the formation of complexes between phosphorylated Smad2 and Smad4 [20,21]. At that time, mutation of Asp-537 had not been found in human tumours, although the equivalent residue in Smad2 (Asp-450) had [21]. These observations led to the idea that Smad2 and Smad4 form a heterodimer in which the loop–helix region of Smad4 interacts with the three-helix bundle region of Smad2 [21]. The more recent identification of tumour cell lines in which either Asp-537 or Asp-351 are mutated (SW948 and CACO-2 respectively) [19] suggested that, *in vivo*, both these residues are critical for Smad4 function. We therefore investigated the functional consequences of these Smad4 point mutations by studying the endogenous mutated Smad4s in the tumour cells in which they naturally occur and also by expressing Smad4 variants bearing these mutations in other TGF-β-responsive cell lines.

Our results indicate that in the tumour cells expressing these mutated Smad4s, the receptors are activated normally in response to TGF-β, since Smad2 becomes phosphorylated and translocates to the nucleus on TGF-β induction. However, the point-mutated Smad4s are not capable of interacting with endogenous-activated Smad2 in an IP/Western-blot assay or with Smad3 in a DNA-binding complex on the c-*Jun* SBR. As a result of their inability to bind activated R-Smads, these Smad4s do not accumulate in the nucleus on TGF-β induction. We also show in transfection assays that the point-mutated Smad4s cannot be incorporated into an ARF1 complex with activated Smad2 and XFoxH1a and they cannot activate transcription from reporters driven by Smad2–Smad4 (ARE_3-luciferase) or Smad3–Smad4 ($CAGA_{12}$-luciferase) complexes. We therefore conclude that both of these point-mutated Smad4s are non-functional. Consistent with this, endogenous transcriptional responses to TGF-β in the SW948 cells and CACO-2 cells are severely disrupted, and both cell lines are resistant to TGF-β-induced growth arrest.

Taken together, our results indicate that in Smad4, both Asp-351 and Asp-537 are required for complex formation with both activated Smad2 and Smad3. For Smad2, recent work [10] has suggested, at least in the context of the ARF complexes with FoxH1 family members, that the Smad2–Smad4 complex is a trimer. Given that the interface between adjacent monomers in a homotrimer of phosphorylated Smad2 MH2 domains is highly

homologous with the interface between monomers in the homotrimer of Smad4 MH2 domains (Figure 6) [21,35], we assume that the same residues (in particular, Asp-351 and Asp-537 of Smad4) will be critical for interaction between adjacent subunits in a Smad2–Smad4 heterotrimer. For Smad3, the results were more surprising, since recent work [10] has suggested that, *in vivo*, Smad3 and Smad4 form a heterodimer in the context of the DNA-bound Smad3–Smad4 complex on the c-*Jun* SBR. If the Smad3–Smad4 heterodimer has a structure similar to that proposed for a Smad2–Smad4 heterodimer [21], we predict that mutation of Asp-351 would abolish interactions between Smad3 and Smad4, but mutation of Asp-537 would not. However, our observation that no Smad3–Smad4 complex is formed on the c-*Jun* SBR in either CACO-2 or SW948 cells, coupled with the observation that overexpression of Smad4 D351H or D537Y cannot rescue the Smad4 deletion in MDA-MB468 cells to activate transcription of the $CAGA_{12}$-luciferase reporter mediated through Smad3–Smad4 complexes, strongly suggests that both Asp-537 and Asp-351 in Smad4 are absolutely required for complex formation with activated Smad3. It is clear that the Smad3–Smad4 complex that binds the c-*Jun* SBR contains at least two additional proteins, and these are necessary for the formation of a stable complex [10]. The most probable explanation for the requirement of Asp-537 for Smad3 interaction is therefore that binding of one of these proteins requires this residue for interaction. We are currently investigating this.

Our results also shed light on TGF-β-induced Smad translocation and thus TGF-β signalling. The current model is that, in the cytosol, phosphorylated R-Smads form complexes with Smad4, which accumulate in the nucleus [1]. In the present study, we confirm that complex formation with Smad4 is not necessary for activated R-Smads to accumulate in the nucleus, as demonstrated previously in different systems [26,36]. However, we show that Smad4 is necessary for the formation of stable Smad–transcription factor complexes on DNA and for transcriptional activation. The most striking observation is our demonstration that for endogenous Smad4 to accumulate in the nucleus in response to TGF-β, it must be complexed with activated R-Smads. We show that it is not the Smad4 import/export that is affected by the MH2 domain mutations, since the CRM1 inhibitor LMB is capable of inducing the nuclear accumulation of both wild-type and mutant Smad4s. This demonstrates clearly that all of them shuttle between the cytoplasm and the nucleus in the basal state, presumably as monomers. However, for Smad4 to accumulate in the nucleus on TGF-β signalling, it must be complexed with activated R-Smads. There are several possible explanations for this, which are not mutually exclusive. First, R-Smad–Smad4 complexes may be actively retained in the nucleus through interactions with DNA or with other proteins, whereas monomeric Smad4 is not [3]. Alternatively, these complexes may be retained in the nucleus, since complex formation leads to masking of the nuclear export signal of Smad4 [6], resulting in Smad4 import being dominant over export, and thus to a preferential nuclear localization. A third possibility is that complex formation in the cytoplasm may lead to enhanced nuclear import, which again would result in import being dominant over export. More work is required to investigate fully these different possibilities.

In conclusion, studying naturally occurring Smad4 mutants *in vivo* in tumour cells has given us insights into the mechanism of Smad4 action. In contrast with conclusions drawn from *in vitro* studies, we have shown that Smad4 function is abolished by these point mutations. Our results have shed light on Smad complex formation after TGF-β stimulation and its requirement for Smad4 nuclear accumulation and transcriptional regulation. This knowledge will in turn contribute to an understanding of how aberrant TGF-β signalling leads to the onset and progression of cancer in different tissues.

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